cant tachyphylaxis of the response. Antagonist experiments were performed on responses induced with 100 nM SP, a concentration that produced a submaximal response.

CP-96,345 (32, 100, or 1000 nM) antagonized SP-induced excitation of locus ceruleus neurons (Fig. 3, B and C), reducing the firing rate increases produced by 100 nM SP in 14 of 14 neurons recorded from 13 slices. The median inhibition concentration (IC_{50}) for the antagonism of SP responses by CP-96,345 was 90 nM. CP-96,345 (32 to 1000 nM) alone did not significantly change baseline firing rates of locus ceruleus neurons (control, 0.37 ± 0.08 Hz; CP-96,345, 0.36 ± 0.07 Hz, n = 14). The (2*R*,3*R*)-enantiomer of CP-96,345 did not have significant activity at 1000 nM, reducing the excitatory response to 100 nM SP by less than 10% in the two neurons examined. An NK_1-specific action of CP-96,345 was indicated by the compound's inability to antagonize the excitations produced by the NK_3-specific agonist senktide (*15*). Excitations induced by 1 nM senktide, which elicited an excitatory effect equivalent to 100 nM SP, were inhibited 5% by 1000 nM CP-96,345 in three of three neurons. Thus, the ability of CP-96,345 to antagonize SP-elicited responses but not the excitant effects of senktide indicates that locus ceruleus neurons have multiple tachykinin receptors and that the excitatory effects of SP are mediated primarily by NK_1 receptors.

CP-96,345 is a nonpeptide compound that acts as a potent and selective NK_1 antagonist in brain. Its pharmacological characteristics make it an important tool for elucidating the physiological significance of NK_1 receptors. In addition, CP-96,345 may provide the means for determining the usefulness of an NK_1 receptor antagonist as therapy for disorders of the nervous system.

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A Kinetic Partitioning Model of Selective Binding of Nonnative Proteins by the Bacterial Chaperone SecB

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An in vitro assay for the interaction of SecB, a molecular chaperone from *Escherichia coli*, with polypeptide ligands was established based on the ability of SecB to block the refolding of denatured maltose-binding protein. Competition experiments show that SecB binds selectively to nonnative proteins with high affinity and without specificity for a particular sequence of amino acids. It is proposed that selectivity in binding is due to a kinetic partitioning of polypeptides between folding and association with SecB.

MOLECULAR CHAPERONES ARE proteins whose general function is to ensure the correct interactions within and between other polypeptides (*1*). Chaperones are involved in the assembly of oligomeric proteins (*2, 3*), in ensuring that certain proteins fold correctly (*4*), in facilitating protein localization (*3, 5*), and in the prevention of formation of proteinaceous aggregates during physiological stress (*6*). Since a single chaperone commonly interacts with a number of different polypeptides, how a chaperone specifically recognizes and binds to a target is an important and intriguing question.

SecB, an oligomeric molecular chaperone in *E. coli*, is involved in export of a subset of unrelated proteins (*7–9*). SecB not only enhances the efficiency of delivery of the precursors to the membrane (*10*), most likely through specific interaction with SecA (*11*), but also is responsible for maintaining the proteins in a loosely folded state so that they remain competent for translocation from the cytoplasm across the cytoplasmic membrane to their final destination in either the periplasmic space or in the outer membrane (*9–11*). The interaction of cytoplasmic SecB with precursor maltose-binding protein (MBP), one of the proteins that depends on SecB for efficient export, occurs early in the export pathway in vivo (*7*) but does not involve recognition of the amino-terminal leader sequence [(*12–14*); see (*15*) for an opposing view]. SecB binds the precursor polypeptide at sites that are distinct from the leader sequence (*16*). The studies described here indicate that precursor MBP contains no unique sequence of amino-acyl residues that serves as a hallmark for binding of SecB. SecB bound tightly to all nonnative polypeptides tested even though it did not interact with any native protein. It seems that SecB has little selectivity for the sequence of amino-acyl residues.

If SecB recognizes all nonnative structures, how are proteins that are to be exported distinguished from those that are to remain in the cytoplasm? We propose that selectivity results from a kinetic partitioning. The pathway a polypeptide takes in vivo depends on the rate of its folding relative to the rate of its association with SecB. Proteins that rapidly fold are precluded from binding, whereas the presence of a leader,

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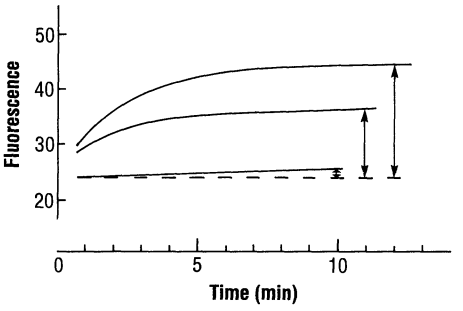

Fig. 1. Effect of SecB on folding of MBP. Three traces of the change in fluorescence of precursor MalE14-1 (concentration 52 nM) with time after initiation of the folding reaction in the presence of 0, 200, or 600 nM SecB at 25°C are shown. The final amplitudes of the fluorescence changes are indicated by the double-headed arrows, the longest indicating the amplitude in the absence of SecB and the shortest that in the presence of 600 nM SecB. In order that all three traces should start at the same fluorescence, given by the dashed line, the fluorescence due to SecB and SecB binding (*14*) has been subtracted. The various forms of MBP and SecB were purified as described (*16, 20*). Measurements of Trp fluorescence were made with a Shimadzu RF-540 fluorescence spectrophotometer with excitation and emission wavelengths of 295 nm (bandwidth, 2 nm) and 344 nm (bandwidth, 5 nm), respectively. Purified MBP was unfolded by incubation for 2 hours at room temperature in 2 M GuHCl buffered with 10 mM Hepes (pH 7.6). Refolding was initiated by rapid addition of the unfolded protein to 2.7 ml of a solution containing a known concentration of SecB in the same buffer without GuHCl held at 25°C in a cuvette in the chamber of the spectrophotometer. The sudden reduction in the concentration of GuHCl from 2 to 0.055 M initiates the folding reaction. The concentrations of proteins in these studies were measured (*34*) with bovine serum albumin as a standard except where indicated otherwise.

which has been shown to retard folding of precursor polypeptides (*17*), would favor association with SecB and thus the pathway leading to export. It has been proposed that other functions attributed to chaperones may also involve a kinetic partitioning among possible pathways (*18, 19*).

The interaction of SecB with MBP can be studied in vitro with the use of the purified proteins since binding of SecB to denatured MBP blocks its folding. The interaction is assessed by monitoring the change in the intrinsic fluorescence of tryptophanyl residues as denatured MBP refolds (*20*). The procedure is briefly described here. MBP is unfolded by incubation with guanidinium chloride (GuHCl). The denaturant is then rapidly diluted, and the change in fluorescence is monitored with time. As MBP folds, side chains of tryptophanyl residues that were exposed to solvent in the unfolded state become buried; thus, one can follow refolding by observing the progressive increase in the amplitude of the fluorescence signal. Two phases in the increase of fluorescence intensity are observed: an immediate increase, which occurs on a time scale below the resolution of our technique (*14*), and a slower phase, which reflects the folding reaction under study here (Fig. 1).

When denatured MBP was diluted into a solution containing an excess of SecB, immediate and complete blockage of refolding occurred provided conditions were chosen so that refolding was appropriately slow (*14*). If refolding was sufficiently rapid, the rate of folding was altered but a stable blockage was not observed. Under the conditions in which an excess of SecB effected immediate and complete blockage, partial blockages were achieved when SecB was present at lower molar ratios. Blockage was not affected by the presence of 5 mM adenosine triphosphate (ATP). The magnitude of the blockage caused by SecB was calculated from a comparison of the amplitude of the change in fluorescence that reflects the slow-folding reaction in the presence of SecB with the same parameter determined in the absence of SecB (Fig. 1). Such analyses (Fig. 2, A and B) revealed a sigmoidal dependence of the blockage of folding on the concentration of SecB whether the MBP used was the precursor or the matured species.

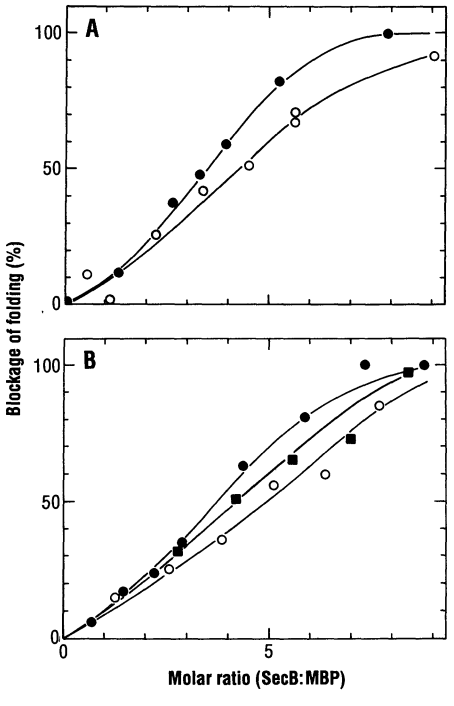

Fig. 2. Blockage of folding of MBP by SecB. (**A**) Wild-type mature MBP at (●) 52 and (○) 4.6 nM at 5°C. (**B**) Wild-type precursor (48 nM) MBP at (■) 10°C and MalE14-1 precursor (52 nM) (●) at 10° and (○) 25°C. Blockage of folding was carried out as described in the legend to Fig. 1. The concentration of wild-type precursor MBP was estimated by densitometric scanning of stained polyacrylamide gels with mature MBP as a standard. The molecular weights used to calculate the molar concentrations of the proteins were 16,600 for SecB, 40,700 for mature MBP, and 43,400 for precursor MBP (*35*).

Since SecB is an oligomer, the sigmoidal shape of the curve might reflect the cooperativity of formation of the active oligomeric species. However, oligomerization of SecB would be expected to be a cooperative function of the absolute concentration of SecB and thus this explanation is inconsistent with the observation that when the concentration of MBP was changed by a factor of 10 the same level of blockage was achieved, not at the same absolute concentration of SecB but at approximately the same molar ratio of SecB to MBP (Fig. 2A).

An alternative explanation for the sigmoidal dependence of blockage of folding on the concentration of SecB is that blockage is effected by the interaction of more than one SecB oligomer with MBP. Consistent with this idea are investigations with hybrid proteins that have implicated two distinct regions of MBP as sites of interaction with SecB (*12, 13*). If SecB does bind to multiple sites on MBP there is little selectivity for specific amino-acyl residues in the binding sites or the recognized amino-acyl sequence is very short. Further support for this contention comes from the demonstration that several unrelated polypeptides bind to SecB.

The presence of polypeptides that compete with MBP for association with SecB reduces the blockage of the folding of MBP exerted by SecB. This reduction in the blockage can be used as an assay for the binding of the competing species. Since folding of MBP was monitored by changes in the fluorescence of tryptophan, proteins which contain no tryptophan were chosen as competitors. Two of the proteins used, bovine pancreatic trypsin inhibitor (BPTI) and pancreatic ribonuclease A (RNase A), have a property that simplifies interpretation of results: they remain in a nonnative state as long as a reducing agent is present (*21*). The other two proteins tested, ribose-binding protein (RBP) and the α subunit of tryptophan synthase, cannot easily be maintained in a nonnative state and refold during the course of the experiment. The form of RBP used has a change in its primary structure that slows the folding and accelerates the unfolding (*22*), thus reducing the stability of the native protein. The α subunit of tryptophan synthase from *E. coli* was chosen because its folding pathway has been well studied by Matthews and his colleagues (*23*) and it is not an exported protein.

In order to determine the affinity for SecB

of the competing species relative to that of MBP in a given experiment, the ratio of SecB to MBP was held constant. The extent of blockage in the presence of the competitor was expressed as a fraction of the blockage in the absence of the competitor for each experiment. In all cases as the quantity of denatured competitor included in the assay increased, the ability of SecB to block the folding of MBP decreased (Fig. 3). Denatured BPTI and denatured RNase A were the most effective competitors. Denatured RBP was the least effective, and the denatured α subunit of tryptophan synthase showed an intermediate level of competition.

The native forms of BPTI, RNase A, and the α subunit of tryptophan synthase showed no competition for binding of SecB. The highest levels tested were molar excesses over MBP of 40, 40, and 55, respectively. The mutant form of RBP, which had not been denatured, was approximately half as efficient in competition with MBP as was the denatured form. Since 0.055 M GuHCl was present in all competition experiments and since the mutant form of RBP is unstable, a significant fraction of the protein may have been in an unfolded state. To demonstrate that it was unfolded RBP that caused the observed competition, 2 mM ribose was included in all buffers to stabilize the native state of the mutant protein. Under those conditions the competition exerted by the nondenatured protein decreased by a factor of 10.

We conclude that SecB specifically binds nonnative proteins. Since it does not interact with native proteins, SecB could not catalyze unfolding, although it could shift the equilibrium between the native and nonnative states of a protein by binding any nonnative polypeptides present.

The degree of competition exhibited by denatured BPTI and denatured RNase A was the same whether the SecB was incubated with the competing species before unfolded MBP was added or the competing species was added to SecB simultaneously with the unfolded MBP (Fig. 3). The data gathered in these competition experiments allow one to calculate relative, apparent affinities of SecB for the proteins if the dissociation constant for one of the complexes is known. We determined this value for nonnative BPTI by using as an assay of binding the change in the fluorescence of SecB caused by the association of SecB, which has a single tryptophanyl residue, with reduced carboxamidomethylated BPTI (Fig. 4). Independent determinations were performed at different concentrations of SecB, at temperatures varying from 5° to 15°C, and with BPTI as the invariant component instead of SecB. Under all conditions the values obtained for the dissociation constant fell within a range of 3 to 8 nM. We used a value of 5 nM and estimates of the relative binding affinities of SecB for MBP and for denatured BPTI obtained from competition experiments (see legend to Fig. 3) to calculate an apparent dissociation constant for MBP of ~1.5 nM. The corresponding calculations for the other competitors yielded relative, apparent dissociation constants of 8, 20, and 50 nM for RNase A, the α subunit of tryptophan synthase, and RBP, respectively. Native BPTI did not interact with SecB in either the direct binding assay or the competition assay. The highest concentration used in the binding assay allows us to estimate that the dissociation constant must be 4 μM or greater. This result explains the lack

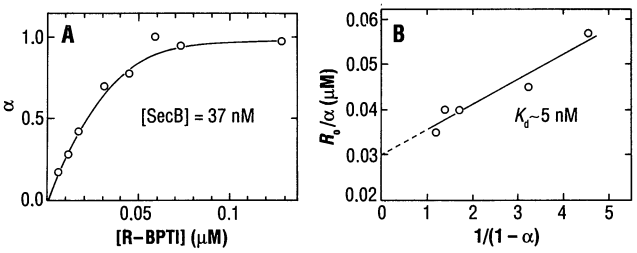

Fig. 4. Determination of the dissociation constant of the complex between SecB and carboxamidomethylated-BPTI (R-BPTI). The binding curve was generated by making successive additions of R-BPTI, dissolved in 10 mM HCl, to a cuvette held at 5°C containing 37 nM of SecB in 10 mM Hepes, pH 7.6, 10 mM DTT, and recording the increase in fluorescence as soon as a constant value was attained, usually within 2 min of the addition. The settings of the fluorescence spectrophotometer were the same as in Fig. 1 except that the emission bandwidth was 20 nm. The increase in fluorescence was plotted as the fraction (α) of the maximal fluorescence increase obtained versus the concentration of ligand added (R_0). The dissociation constant of the complex between R-BPTI and SecB was obtained from the binding curves by plotting R_0/α against $1/(1 - \alpha)$. For a simple two component binding reaction this graph gives a straight line with a gradient equal to the dissociation constant and an intercept at the ordinate equal to the concentration of binding sites in the invariant component (SecB in this case) (*38*).

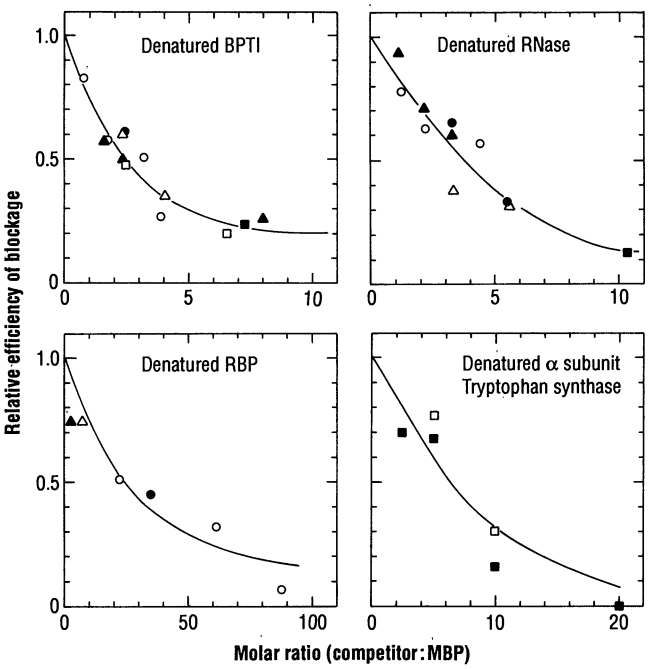

Fig. 3. Competition for binding of SecB between nonnative MBP and four other denatured proteins. Experiments were conducted at 5°C as described in the legend to Fig. 1 in the presence and absence of denatured competitor. The ratio of SecB to mature MBP was chosen such that blockage in the absence of competitor would be in the steep part of the sigmoid curve (40 to 80% blockage). The relative efficiency of blockage is the blockage obtained in the presence of the competitor expressed as a fraction of that obtained in its absence. Different symbols within each panel indicate different experiments. The circles and squares in the upper two panels are from experiments in which the competitor was added to the cuvette before the MBP. In all other experiments the two proteins were added simultaneously. BPTI and RNase A were denatured by incubation at room temperature for 2 hours in 5.4 M GuHCl, 10 mM dithiothreitol (DTT), 1 mM EDTA, 10 mM Hepes, pH 7.6; the α subunit of tryptophan synthase was denatured by incubation for 2 hours at room temperature in 2 M GuHCl, 10 mM DTT, 10 mM Hepes, pH 7.6. For these three proteins the competition was conducted in the presence of 10 mM DTT. A slow-folding form of RBP, RbsB V50E (*22*), was denatured by incubation for 1 hour at room temperature in 0.4 M GuHCl, 10 mM Hepes, pH 7.6. The molecular weights used to calculate molar concentrations were 6,500 for BPTI, 13,700 for RNase A, 29,800 for RBP, and 28,700 for the α subunit of tryptophan synthase (*36*). The concentration of the α subunit of tryptophan synthase was determined spectroscopically by using a specific absorption coefficient $\varepsilon^{1\%}_{278\ nm} = 4.4$ (*37*). The relative affinities of SecB for MBP and a competitor were calculated as follows. If the presence of an n-fold molar excess of competitor over MBP reduces the blockage of folding at the true molar ratio z (SecB:MBP) to a value characteristic of a lower molar ratio a, then $nR + 1 = z/a$ from which R may be calculated (R is the reciprocal of the ratio of the dissociation constants of the SecB complexes).

of competition by native BPTI and reinforces the conclusion that SecB does not interact with fully folded proteins. Lecker *et al.* (*24*) demonstrated that when diluted from urea, proOmpA and prePhoE, the precursors of two outer membrane proteins, form stable isolable complexes with SecB. Stable complexes were not detected between SecB and RNase A or with several other soluble proteins. The authors point out that these proteins might initially associate with SecB, but their folding would internalize the binding sites, thus causing association to be transient. This interpretation is consistent with our observation that only nonnative proteins interact with SecB.

SecB has similar affinities for all nonnative ligands tested; however, it must be emphasized that binding of SecB to the ligands was not determined directly but the relative affinities were estimated by competition. It is impossible to assign precise values for these affinities because as stated, three of the polypeptides, MBP, RBP, and the α subunit of tryptophan synthase, refold during the course of the competition experiments. If our model is correct, as a protein folds the binding sites for SecB would become inaccessible, resulting in a progressive decrease in the concentration of the binding sites, and would thereby complicate quantitative interpretation of the measurements. Nevertheless, it is clear that SecB recognizes a wide range of polypeptides in addition to its physiological ligands, including mammalian secretory proteins and, from *E. coli*, a cytosolic protein and a periplasmic protein (RBP) that does not require SecB for export. Since a search for sequence similarities among the five proteins revealed nothing of significance [computer programs used were COMPARE and DOTPLOT from the UWGCG software (*25*)], recognition probably occurs with little selectivity beyond that for a nonnative state. If the binding site is masked in native proteins, selectivity of the binding of SecB to proteins would be greatly influenced by the rate at which a polypeptide folds.

As indicated previously, the folding of wild-type mature MBP could be completely blocked only when the rate of folding was sufficiently slow. All other species of MBPs tested formed stable complexes with SecB provided that temperatures were chosen so that each species refolded approximately as slowly as did the wild-type mature species at 5°C (the temperature of the blockage experiment shown in Fig. 2A): for wild-type precursor, 10°C (Fig. 2B); for precursor with an altered leader sequence, 10°C (Fig. 2B, precursor MalE14-1); and for a slow-folding mature species, 25°C (Fig. 5, MalEY283D). For any given species of MBP the amount of SecB necessary to exert a particular level of blockage of folding was shifted to higher values if the protein was allowed to refold more rapidly [compare precursor MalE14-1 refolding at 10°C with refolding at 25°C (Fig. 2B) and MalEY283D refolding at 5° and 25°C (Fig. 5)]. We believe that the parameter crucial in determining how much SecB is required to block folding is the rate of folding. However, it might also be that the change in temperature alters the affinity of SecB for its binding site. We do not think that this is the case for several reasons. First, the dissociation constant for SecB complexed to another ligand, nonnative BPTI, was shown to be the same over the temperature range tested, 5° to 15°C. Second, competition experiments carried out between nonnative BPTI and wild-type precursor or precursor MalE14-1 at 10°C or wild-type mature MBP at 5°C gave the same relative affinity of SecB for the three species of MBP (*26*). Finally, the blockage of folding experiments are done with the concentrations of SecB between 50 and 500 nM, well above the estimated dissociation constant of 1.5 nM, and it would therefore require a dramatic change in dissociation constant to change the extent of binding significantly.

The correlation between the increase in rate of folding and need for increased quantities of SecB to block folding can be explained simply if folding masks the binding site for SecB. As the rate of folding increases the probability that at any moment a polypeptide has its binding site exposed decreases. When the amount of SecB is increased the probability that it will bind, which is a function of the rate of association and the concentration of SecB, is higher. The slowest folding that could be achieved experimentally, that of the slow-folding species MalEY283D (*14*) at 5°C, was completely blocked by SecB at a molar excess of ~4 over MBP (Fig. 5).

SecB might recognize nonnative structures during the course of folding of polypeptides by interacting with intermediates that exist transiently. The interaction is probably not specific for a given intermediate in a folding pathway; most likely the elements recognized are areas of hydrophobicity or regions of extended polypeptide chain that could form hydrogen bonds from the backbone to the binding site on the SecB oligomer that itself might contain an extended β strand. Although binding of SecB to a ligand does not strictly require a certain sequence of amino acids, clearly some binding sites are preferred over others since the relative affinity of SecB for the nonnative polypeptides tested varied 50-fold. The observed difference in relative affinity between

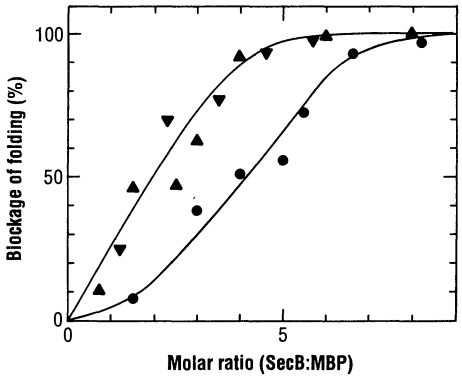

Fig. 5. Blockage of folding by SecB of a slow-folding form of mature MBP. Blockage was carried out at (●) 25° and at (▼ and ▲) 5°C. The concentration of MalEY283D was 50 nM. The two orientations of the triangles indicate different experiments.

MBP and RBP might be sufficient to account for the observation that MBP can be isolated in a complex with SecB whereas only minimal amounts of RBP, which does not depend on SecB for efficient export, are found complexed with SecB in vivo (*27*). However, since the affinity of nonnative protein for SecB is high, we propose that the selectivity in binding of ligands in vivo by SecB not only is a function of the affinity but in addition is modulated by a kinetic partitioning between the pathways of folding and association. It has been proposed that in vivo the leader peptide of precursor MBP slows the folding and favors binding to SecB and subsequent export (*14, 17, 28*). Let us consider the feasibility of such a partitioning in vivo. The best estimate available for the rate constant of folding of precursor MBP at 30°C is between 0.015 and 0.25 s^{-1} (*29*). If we assume that the association is diffusion-limited, a reasonable estimate for the rate constant would be 10^7 M^{-1} s^{-1} (*30*). If 10% of the SecB present in the cell were uncomplexed at any time (*31*), then the pseudo–first-order rate constant for association with SecB would be on the order of 4 s^{-1}. Clearly the precursor species folds slowly enough that the great majority would bind to SecB before it could fold. Rate constants for folding of cytosolic proteins are expected to be of the order of 5 to 10 s^{-1} (*32*) so that for these polypeptides the partitioning would favor folding. In addition, the precursors would be rapidly exported whereas cytosolic proteins lacking a leader peptide would not cross the cytoplasmic membrane, and if they did bind to SecB they would repartition until the entire population was folded.

Since as discussed above the affinity of RBP for SecB is not high enough to compete with other ligands in vivo, we must ask how RBP is maintained in an export-com-

petent state. Ribose-binding protein and other proteins that are exported efficiently in the absence of SecB may use other chaperones or the folding of these proteins may be sufficiently slow that even in the absence of a chaperone they remain in a state that is capable of interaction with the export apparatus.

Kinetic partitioning similar to that described to explain selective entry of precursors into the export pathway might also be the basis of other functions attributed to chaperones. Chaperones may interact with nonnative polypeptides to establish an equilibrium such that the polypeptide would continually reassociate with the chaperone unless it was afforded a more rapid pathway by changes in local conditions. For example, accumulation of another polypeptide might favor proper assembly of an oligomeric protein or transfer to a different subcellular compartment might provide conditions for proper folding. Although SecB is not among them, many chaperones hydrolyze ATP (*18*). This hydrolysis, known in some cases to modulate dissociation (*33*), may serve to control the timing of release so that it is more likely to occur under favorable conditions. SecB could release precursor polypeptides without hydrolysis of ATP by exchange to the next component of the export apparatus. An exchange mechanism is supported by the observation described here that MBP is readily released from SecB by competing species. We propose that, like SecB, other chaperones may be capable of interacting indiscriminately with proteins that have not attained their native structure and that selectivity in binding is imposed by a kinetic partitioning between association and other available pathways.

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29. The complete study of folding of MBP previously published (*20*) shows that under the conditions used in the experiments described here the relaxation time observed reflects a reaction (likely to be isomerization about a peptide bond which contains a Pro residue) that is independent of concentration of denaturant and is rate limiting at concentrations of GuHCl below 0.5 M. At 30°C the rate constant of this reaction for precursor is 0.015 s^{-1}. The rate constant for the folding reaction that is dependent on concentration of denaturant (0.25 s^{-1}) was obtained by extrapolation from relaxation times above 0.5 M GuHCl.
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32. The rate of folding for proteins that do not contain *cis*-prolyl residues is in the 100-ms time range. An estimate for the rate constant of the fast-folding form of RNase at 30°C is 10 s^{-1} [P. J. Hagerman and R. L. Baldwin, *Biochemistry* **15**, 1462 (1976)] and 5 s^{-1} for mature MBP (L. L. Randall, unpublished results). If proteins contain a *cis*-prolyl residue a slow-folding form is also apparent. For mature MBP the rate constant for the slow form is 0.5 s^{-1}. For a review of folding rates, see P. S. Kim and R. L. Baldwin, *Annu. Rev. Biochem.* **51**, 459 (1982).
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